

## Research Article

# Resistance of Platelets in Hypercholesterolemia to Inhibition by Activated Coagulation Factor X

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Platelet hyperactivity may be involved in the pathogenesis of both thrombogenesis and hypercholesterolemia. The cholesterol-enriched states may contribute to accelerated development of atherosclerosis. The effect of high cholesterol on platelet activation and on inhibition by coagulation factor Xa, was studied *in vitro*. Incubation of normal platelets ( $n = 20$ ) with cholesterol-rich dispersion resulted in a small increase of platelet aggregation (PA) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) synthesis when compared with platelets incubated with cholesterol-normal dispersion. In hypercholesterolemic patients ( $n = 20$ ), ADP-induced PA and TXA<sub>2</sub> synthesis showed only small increases over normal controls. Addition of factor Xa (1 unit/mL) prevented the ADP-induced PA and markedly inhibited TXA<sub>2</sub> synthesis in normal platelets ( $1.3 \pm 0.2$  and  $8.7 \pm 2.0$  pmol TXA<sub>2</sub>/10<sup>8</sup> platelets, with and without factor Xa, resp.). However, factor Xa failed to significantly suppress TXA<sub>2</sub> synthesis in cholesterol-incubated normal platelets ( $9.5 \pm 1.4$  and  $11.8 \pm 1.3$  pmol TXA<sub>2</sub>/10<sup>8</sup> platelets, with and without factor Xa; resp.,  $P = \text{NS}$ ) as well as in platelets from patients with hypercholesterolemia ( $8.6 \pm 4.0$  and  $10.9 \pm 4.9$  pmol TXA<sub>2</sub>/10<sup>8</sup> platelets, with and without factor Xa; resp.,  $P = \text{NS}$ ). Exposure of platelets to high cholesterol concentrations, *in vitro* and *in vivo*, marginally increased PA and TXA<sub>2</sub> synthesis but resulted in loss of responsiveness to factor Xa, which could significantly contribute to platelet activation in hypercholesterolemic states.

## 1. Introduction

Activation of platelets at the site of coronary artery plaque disruption is the cause of the acute coronary syndromes. Through recurrent thrombosis and the release of chemotactic and growth factors, platelets contribute to chronic arterial narrowing [1]. Numerous studies have demonstrated an inverse relationship between the risk of coronary artery disease and cholesterol in plasma [1, 2]. Platelets are involved in both the initiation and progression of atherosclerotic lesions [3, 4]. During atherosclerosis, heightened oxidative stress in the artery wall gives rise to oxidized forms of low-density lipoproteins (LDL) that provoke an inflammatory response [4–6]. The enhancement of platelet aggregation by LDL may contribute to the accelerated development of atherosclerosis [7]. In individuals with elevated lipid levels,

increased platelet reactivity is a highly prevalent finding [8, 9]. *In vivo* studies have provided evidence that platelets serve as a rich source of cholesterol that can be accumulated by macrophages and smooth muscle cells and stored as lipid droplets [10]. Furthermore, platelets enhance the rate of cholesterol ester formation and total cholesteryl ester accumulation in cultured peripheral mononuclear-derived macrophages. Thus, the suppression of platelet activity is essential for anti-atherogenesis [11].

Activated blood coagulation factor X, the key protease in the prothrombinase complex (which converts prothrombin to thrombin), has been reported to inhibit aggregation of platelets induced by ADP, thrombin, and epinephrine [12, 13]. Factor Xa (FXa) is a key enzyme that is positioned at the convergence of the intrinsic and extrinsic pathways in the

blood coagulation cascade, and inactivation by a specific FXa inhibitor effectively prevents the generation of thrombin.

Hypercholesterolemia, a long recognized cause of atherosclerosis [14, 15], has been reported, by several investigators, to be associated with platelet hyperaggregability [16, 17] and, experimentally, with microvascular obstruction and enhanced platelet deposition after arterial injury [18, 19]. Other workers have not confirmed these findings [8, 9]. Resistance of platelets to prostaglandins  $E_1$  and  $I_2$  has been proposed as an alternative mechanism of increased platelet activity in hypercholesterolemia [20, 21].

To further characterize the antiplatelet action of coagulation factor Xa and to assess the relative effect of cholesterol on platelet hyperactivity and inhibitor resistance, we studied the behavior of normal platelets, cholesterol-enriched platelets, and platelets from patients with hypercholesterolemia in relation to common agonists and the inhibitory coagulation factor Xa.

## 2. Materials and Methods

**2.1. Patient Selection.** The subjects included normal volunteers ( $n = 20$ ), and patients ( $n = 20$ ) with hypercholesterolemia (hyperlipoproteinemia IIa) who had not been treated with hypolipidemic agents during the last 12 months before the study. The patients were age matched with controls ranging in age from 38–65 years. None of the participants had taken aspirin or other platelet-active medications for at least 14 days prior to the study. The protocol was approved by the Institutional Review Board for Clinical Research at the BVA Medical Center.

**2.2. Collection of Blood and Preparation of Platelet-Rich Plasma.** After obtaining informed consent, blood was collected from an antecubital vein through a 19-gauge needle into a plastic tube and anticoagulated with 3.8% sodium citrate (nine volumes of blood into one volume of sodium citrate). Platelet-rich plasma was obtained by centrifuging citrated blood at  $200 \times g$  for 15 min. at  $23^\circ C$  [22]. Platelet-rich plasma was prepared from blood samples of normal volunteers and from patients with hypercholesterolemia (hyperlipoproteinemia IIa) who had not been treated with hypolipidemic agents.

**2.3. Chemicals.** Cholesterol and L- $\alpha$ -dipalmitoyl phosphatidylcholine were purchased from Sigma Chemical Company, St. Louis, Mo, USA. Radioimmunoassay of TXB<sub>2</sub> was determined by using a commercially available kit (New England Nuclear, Boston, Mass, USA). All other chemicals were obtained commercially (Sigma, St. Louis, Mo, USA). All chemicals were of analytical grade.

**2.4. Incubation of Platelets with Cholesterol Dispersion.** Cholesterol-normal and cholesterol-rich phospholipid dispersions were prepared as described [23]. In brief, cholesterol and L- $\alpha$ -dipalmitoyl phosphatidylcholine (both from Sigma Chemical Company, St. Louis, Mo, USA) were sonicated for one hour in a modified Tyrode's solution (8 gm NaCl, 1 gm NaHCO<sub>3</sub>, 200 mg KCl, 50 mg NaH<sub>2</sub>PO<sub>4</sub>, 1.41 g MgCl<sub>2</sub> in 1

liter of deionized water). A 70-watt sonifier with standard tip was used (Branson Instruments Co., Stamford, Conn, USA). To prevent overheating during sonication, the cholesterol phospholipid mixture was placed in fluted metal containers and kept in icy slurry during sonication. Prior to their use, all dispersions prepared by sonication were centrifuged at  $21,000 \times g$  for 30 minutes at  $0^\circ C$  (Beckman Ultracentrifuge, model L 350). After adjusting the platelet count to  $2 \times 10^8$ /mL, platelet-rich plasma (5 mL) was incubated for 5 hours at  $37^\circ C$  with equal volumes of, either, cholesterol-normal dispersion (cholesterol:phospholipid molar ratio = 1:1), cholesterol-rich dispersion (cholesterol:phospholipid molar ratio = 2.2:1), or Tyrode's solution as described [23].

**2.5. Platelet Aggregation Studies.** Platelet aggregation studies were performed at  $37^\circ C$  in a dual-channel aggregometer. Aggregating agents used were adenosine diphosphate (ADP), L-epinephrine, and collagen. Concentrations of the aggregating agents required for maximum platelet aggregation were determined for all platelet-rich plasma samples. For each normal donor, the same concentrations of agonists were used in aggregation and thromboxane A<sub>2</sub> studies of platelets incubated in Tyrode's solution, cholesterol-normal, and cholesterol-enriched dispersion. Platelets from patients with hypercholesterolemia were studied with concentration of ADP yielding maximum aggregation in all preparations. To determine the effect of factor Xa, the platelet-rich plasma samples were incubated at  $37^\circ C$  for 30 seconds with the coagulation factor Xa, 1 unit/mL (Sigma Chemical Products, St. Louis, Mo, USA), or an equivalent volume of Tyrode's solution, followed by addition of the aggregating agent [22]. At the beginning of each aggregation study, the strip recorder was adjusted to zero with platelet-poor plasma of the individual studied. Tracings were recorded on a strip chart recorder (Chrono-log Corporation, Broomall, Pa, USA).

**2.6. Thromboxane A<sub>2</sub> Radioimmunoassay.** Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) is unstable and readily hydrolyzed to TXB<sub>2</sub>. Therefore, TXA<sub>2</sub> was determined by radioimmunoassay of TXB<sub>2</sub>, using a commercially available kit (New England Nuclear, Boston, Mass, USA). After platelet aggregation with ADP, the plasma fraction was collected in 1.0 mM EDTA in plastic tubes and centrifuged for 5 minutes at  $8,000 \times g$  in an Eppendorf microfuge [22]. The supernatants were collected and stored at  $-40^\circ C$  until determination of TXB<sub>2</sub>.

**2.7. Adenylate Cyclase Assay.** To determine whether adenylate cyclase played a role in platelet inhibition by factor Xa, adenylate cyclase activity of platelet membranes was determined as previously described [22]. In brief, 1.0 mM ATP containing [ $\alpha$ -<sup>32</sup>P]ATP ( $1.2 \times 10^6$  DPM), 2 mM MgCl<sub>2</sub>, 12 mM theophylline, 1 mM creatine phosphate, 1 unit of creatine phosphokinase, and 25 mM Tris-HCl buffer, pH, 7.5 with varying amounts of PGE<sub>1</sub> was incubated in a total volume of 100  $\mu$ L. After incubating platelets with factor Xa, 1 unit/mL, or Tyrode's solution for control, reactions were initiated by the addition of 20  $\mu$ L of platelet membrane suspension containing approximately 150  $\mu$ g protein and incubated at  $23^\circ C$  for 10 min. Reactions were terminated

by the addition of 0.1 ml of 2% sodium dodecyl sulfate. Radioactive cyclic AMP was separated as described [22]. Unlabeled cyclic AMP (1.0 mM) was added to the reaction mixture at termination to facilitate the recovery of the radionucleotide.

**2.8. Statistical Calculations.** One-way analysis of variance (ANOVA) was used to test for overall differences between responses of platelet preparations to agonists and the inhibitor (factor Xa). Subsequently, the Student-Newman-Keuls test was used for comparisons between individual groups. Platelet preparations compared were those of normal donors incubated with Tyrode's solution, cholesterol-normal and cholesterol-enriched dispersion, and platelets from patients with type IIa hyperlipidemia. Results are expressed as means  $\pm$  SD.

### 3. Results

**3.1. Effect of Cholesterol Enrichment of Platelets on Factor Xa-Induced Inhibition of Platelet Aggregation.** Incubation of normal platelets in cholesterol-rich dispersion resulted not only in enhanced platelet aggregation by ADP, but cholesterol-enriched platelets also became resistant to the inhibitory effect of factor Xa when compared to the same platelets incubated in cholesterol-normal dispersion (Figure 1). Similar results were obtained when ADP was substituted by *L*-epinephrine as aggregating agent. However, in the case of collagen, where factor Xa did not inhibit the aggregation of cholesterol-enriched platelets, the protease only modestly inhibited the aggregation of cholesterol-normal platelets compared to ADP and *L*-epinephrine. No enhanced platelet aggregation was noted in cholesterol-enriched platelets using collagen as aggregating agent (Figure 1). A representative example of the effect of coagulation factor Xa on platelets incubated in cholesterol-normal and cholesterol-rich dispersions is displayed in Figure 2. Aggregation of platelets incubated with Tyrode's buffer alone was similar to that of cholesterol-normal platelets (not shown).

**3.2. Effect of Cholesterol Enrichment of Platelets on Factor Xa-Induced Inhibition of TXA<sub>2</sub> Synthesis.** Incubation of normal platelets with Tyrode's buffer or cholesterol-normal or cholesterol-enriched dispersion, had an only marginal, statistically insignificant effect on TXA<sub>2</sub> synthesis (Table 1). Addition of factor Xa markedly inhibited thromboxane synthesis, both in platelets incubated with cholesterol-normal dispersion and with Tyrode's buffer. In contrast, in platelets from the same donors, the coagulation factor failed to inhibit the synthesis of TXA<sub>2</sub> after the PRP had been incubated in cholesterol-enriched dispersion (Table 1).

**3.3. Effect of Factor Xa on Platelet Aggregation and TXA<sub>2</sub> Synthesis in Hypercholesterolemia.** To determine whether exposure of platelets to a high cholesterol environment *in vivo* results in loss of responsiveness to factor Xa similar to that following platelet cholesterol enrichment *in vitro*, we studied platelets from patients with hypercholesterolemia.

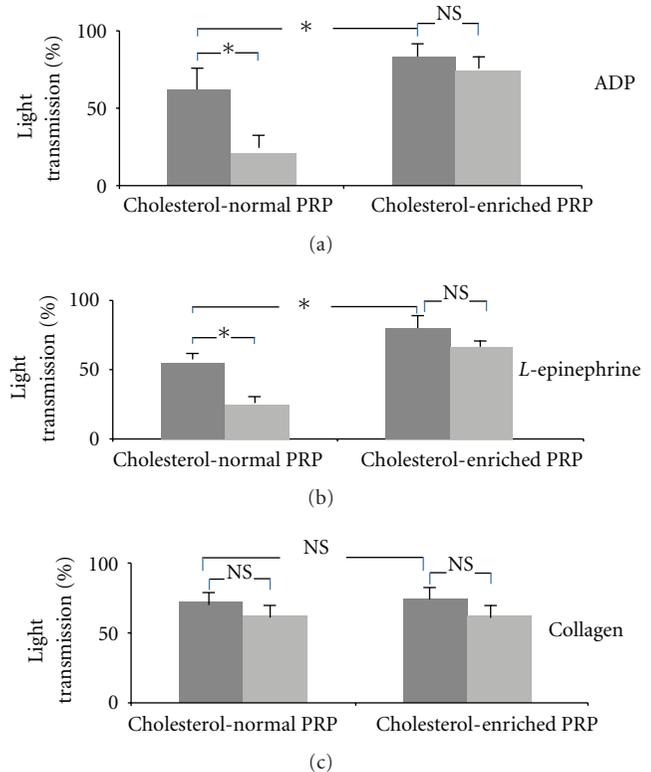


FIGURE 1: Effect of coagulation factor Xa on aggregation of normal and cholesterol-enriched platelets. Platelets from identical normal donors were studied after platelets were incubated in cholesterol-normal (left) and cholesterol-rich (right) phospholipid dispersion ( $n = 20$ ) (details in METHODS). The effect of a 30 sec exposure of platelets to Tyrode's buffer with (dark grey) and without (light grey) factor Xa (1 unit/mL) on aggregation by ADP (a), epinephrine (b), and collagen (c). \* $P < 0.05$ , NS nonsignificant, PRP platelet-rich plasma.

TABLE 1: Effect of factor Xa on ADP-induced thromboxane A<sub>2</sub> synthesis in normal and *in vitro* cholesterol-enriched platelets.

PRP in	Addition	TXA <sub>2</sub> (pmol/10 <sup>8</sup> platelets)	
Tyrode's buffer ( $n = 12$ )	Tyrode's buffer	8.7 $\pm$ 2.0	$P < 0.05$
	Factor Xa	1.3 $\pm$ 0.2	
Cholesterol-normal dispersion ( $n = 12$ )	Tyrode's buffer	9.9 $\pm$ 1.4	$P < 0.05$
	Factor Xa	2.0 $\pm$ 0.3	
Cholesterol-rich dispersion ( $n = 12$ )	Tyrode's buffer	11.8 $\pm$ 1.3*	$P = NS$
	Factor Xa	9.5 $\pm$ 1.4	

\*NS compared to PRP in Tyrode's and cholesterol-normal dispersion. NS: nonsignificant.

The plasma cholesterol profile of these patients is shown in Table 2. As shown in Table 3, platelets from hypercholesterolemic subjects, similar to *in vitro* cholesterol-enriched platelets, exhibited an only small but statistically significant increase in aggregation compared to normal controls (83  $\pm$  8% compared to 62  $\pm$  13%,  $P < 0.05$ ). Addition of

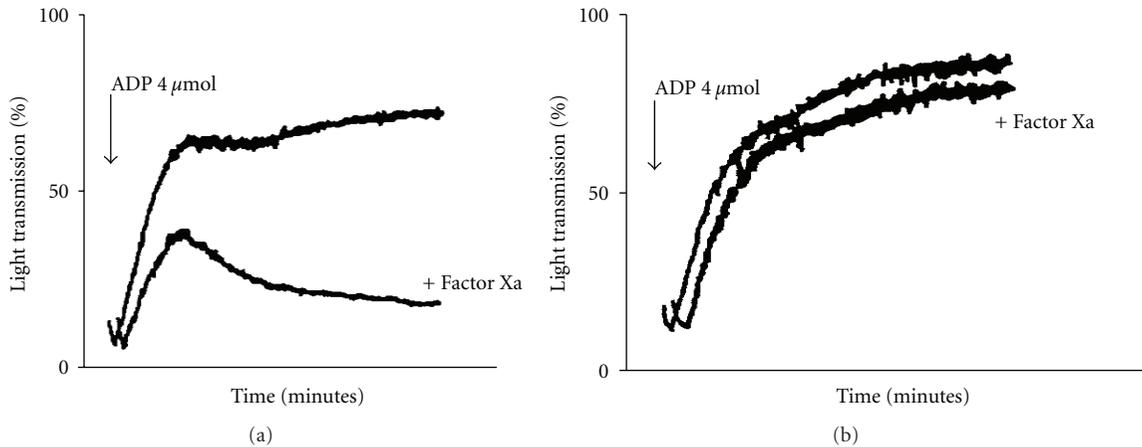


FIGURE 2: Inhibition of ADP-induced platelet aggregation by factor Xa. Aggregation of platelets from the same patient was studied after incubation in cholesterol-normal (a) and cholesterol-enriched (b) phospholipid dispersion as described in the Material and Methods section. Factor Xa concentration was 1 unit/mL.

TABLE 2: Total, low-density (LDL), and high-density (HDL) plasma cholesterol concentration in normal volunteers and hypercholesterolemic subjects.

	Normal controls ( <i>n</i> = 12)	Hypercholesterolemic subjects ( <i>n</i> = 12)
Total cholesterol (mg/dL)	187.5 ± 42.5	301.8 ± 31.3*
LDL cholesterol (mg/dL)	106.0 ± 32.1	192.3 ± 30.4*
HDL cholesterol (mg/dL)	50.1 ± 11.5	69.5 ± 24.3 <sup>+</sup>

\**P* < 0.001 compared to normal controls.

<sup>+</sup>*P* = nonsignificant compared to normal controls.

TABLE 3: Inhibition of ADP-induced platelet aggregation by factor Xa in normal volunteers and hypercholesterolemic patients.

Platelet-rich plasma	Addition	Platelet aggregation %	
Normal controls ( <i>n</i> = 12)	ADP + Tyrode's buffer	62 ± 13	<i>P</i> < 0.05
	ADP + factor Xa	21 ± 20*	
Hypercholesterolemic subject ( <i>n</i> = 19)	ADP + Tyrode's buffer	83 ± 8	<i>P</i> = NS
	ADP + factor Xa	74 ± 15	

\* reversible aggregation only.

NS: nonsignificant.

factor Xa to platelet-rich plasma from patients with hypercholesterolemia reduced ADP-stimulated aggregation only marginally (from 83 ± 8% to 74 ± 15%, *P* = nonsignificant). In contrast, secondary aggregation of platelets from normal donors was completely suppressed by the presence of factor Xa. Primary (reversible) platelet aggregation (with 21 ± 20% increase in light transmission) was not affected by the

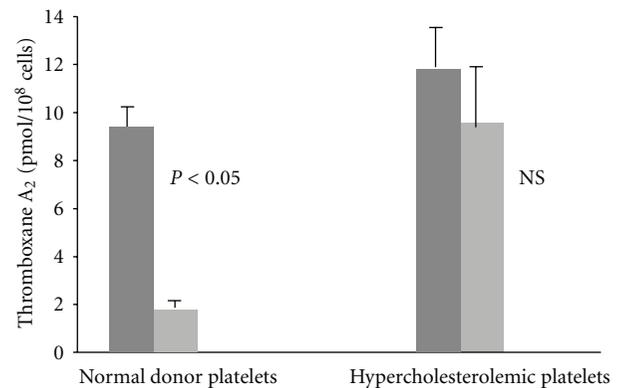


FIGURE 3: Resistance of platelets from patients with hypercholesterolemia to factor Xa. The ADP-induced synthesis of TXA<sub>2</sub> in platelets from normal volunteers (*n* = 20) and patients with hypercholesterolemia (*n* = 20) was determined. Platelet-rich plasma was studied after incubation in Tyrode's buffer with factor Xa, 1 unit/mL, (dark grey) and without (light grey). NS nonsignificant.

coagulation factor (Table 3). As in the case of cholesterol-enriched platelets, where factor Xa failed to inhibit TXA<sub>2</sub> synthesis, platelets from patients with hypercholesterolemia were resistant to the inhibitory effect of factor Xa when compared to normal controls (Figure 3).

#### 3.4. Effect of Factor Xa on Platelet Adenylate Cyclase Activity.

Incubation of platelets with factor Xa (1 unit/mL) did not result in the activation of adenylate cyclase compared with the platelets incubated with Tyrode's buffer. The basal adenylate cyclase activity of control membrane, which was 21 ± 5 pmol/mg protein/10 min, cyclic AMP formed remained essentially unchanged after these cells were treated with factor Xa (25 ± 8 pmol/mg protein/10 min, *n* = 12, *P* = NS).

#### 4. Discussion

The results presented herein demonstrate that *in vitro* cholesterol-enriched platelets and platelets from patients with hypercholesterolemia were resistant to the inhibitory effects of factor Xa, a potent endogenous platelet antagonist [12, 13]. While *in vitro* cholesterol enrichment of platelets induced only a modest increase in TXA<sub>2</sub> synthesis compared to normal platelets, the factor Xa-induced inhibition of TXA<sub>2</sub> synthesis decreased from 85% inhibition in normal donor platelets to 20% inhibition in cholesterol-rich platelets, a fourfold reduction in the inhibitory effect of the protease. A similar loss of responsiveness toward factor Xa occurred in platelet-rich plasma from patients with hypercholesterolemia (Table 3 and Figure 3). Furthermore, the effect of factor Xa on *ex vivo* platelet aggregation paralleled the inhibition of thromboxane synthesis. The effect of factor Xa on platelets, which on a mole per mole basis compares well with PGI<sub>2</sub> [12], was specific and independent of changes in adenylate cyclase activity as shown by failure of the coagulation factor Xa to increase platelet membrane activity of adenylate cyclase in all platelet preparations. These findings are in agreement with our previous finding of unchanged cyclic AMP levels in platelets exposed to factor Xa [12, 13].

High plasma cholesterol levels have long been linked to abnormal platelet reactivity. Incubation of normal PRP with a cholesterol-enriched phospholipid dispersion (molar ratio 2 : 1) increases platelet membrane cholesterol by 55% and has been reported to enhance platelet aggregation and thromboxane synthesis by epinephrine, ADP, thrombin, and the calcium ionophore A 23187 [23–25]. In contrast, the platelet cholesterol/phospholipid ratio, which remains unchanged after incubation in “cholesterol-normal” phospholipid dispersion (molar ratio 1 : 1), does not appear to significantly affect platelet activity [23]. It has been suggested that an apparent increase of platelet hyperactivity in cholesterol-enriched environments may be caused by a decrease in the fluidity of the membrane phospholipid bilayer [26] or enhanced thromboxane synthesis [24, 25]. In addition, native and oxidatively modified low-density lipoproteins bind to platelet membranes where they can function as direct platelet agonists [27–30]. Our results demonstrate that both *in vitro* cholesterol-enriched platelets and platelets from patients with hypercholesterolemia were significantly more sensitive to aggregation when compared to normal control platelets. TXA<sub>2</sub> synthesis, on the other hand, was not significantly increased in either platelets cholesterol-enriched *in vitro* or platelets from patients with hypercholesterolemia. These results raise the possibility of a thromboxane-independent mechanism as the basis for the modestly increased platelet aggregation observed in cholesterol-enriched environments.

The detection of a state of enhanced platelet activity in patients with hyperbetalipoproteinemia has remained elusive. Although the original observations by Carvalho and Lees on the increased sensitivity of platelets from patients with hypercholesterolemia to ADP, epinephrine, and collagen are supported by some observations [17, 31], other studies have not detected a significant increase in *ex vivo* reactivity

of platelets from patients with hyperbetalipoproteinemia [20, 21]. Similarly, the increase in aggregation of platelets from patients with hypercholesterolemia, while statistically significant compared to normal controls, was only small in our study.

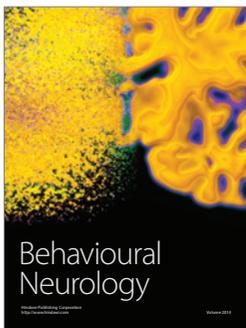
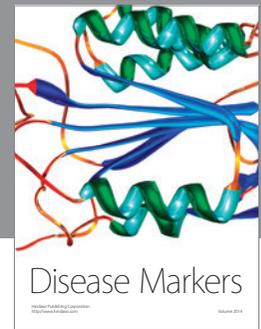
Our results demonstrate that the cholesterol-enriched platelets were resistant to the potent antiplatelet effect of prostaglandins I<sub>2</sub> and E<sub>1</sub> [32, 33]. *In vitro*, low density lipoproteins may impair the antiplatelet properties of cultured endothelium [34] and, through marked decrease in (platelet-inhibiting) endothelium-derived relaxing factor, could potentially decrease the antiplatelet properties of vascular endothelium *in vivo* as well [35, 36]. In this context, the failure of coagulation factor X to inhibit platelet activation in cholesterol-enriched environments may have implications for platelet activation and thrombogenesis in hypercholesterolemia.

*In conclusion*, exposure of platelets to high-cholesterol environments *in vitro* and *in vivo* induced a proaggregatory shift towards platelet activation which resulted in a simultaneously small increase in platelet aggregability and marked failure to the endogenous inhibitor factor X (to inhibit platelet activation) that is essential for thrombin generation. The role of activated coagulation factor X for the regulation of platelet activity *in vivo* and the significance of its failure as a platelet antagonist in hypercholesterolemic states will be determined in our ongoing investigations.

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